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A Spectrophotometric Method for the Assay of Phospholipase D Activity

GEORGE M. CARMAN,[1,*] ANTHONY S. FISCHL,* MAUREEN DOUGHERTY,* AND GERHARD MAERKER†

Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey 08903, and †Eastern Regional Research Center, Agricultural Research, Science and Education Administration, United States Department of Agriculture, Philadelphia, Pennsylvania 19118

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Phosphatidylcholine phosphatidohydrolase (EC 3.1.4.4, phospholipase D) catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. We have developed a spectrophotometric assay for phospholipase D using choline kinase, pyruvate kinase, and lactate dehydrogenase to couple the release of choline with the oxidation of NADH. The assay was linear both with time and with enzyme concentration. The assay should prove useful for continuous monitoring of enzyme activity, determination of initial rates of reaction, and detailed kinetic studies of phospholipase D. The method is limited to analysis of purified preparations of phospholipase D lacking competing activities to the coupled system.

Phosphatidylcholine phosphatidohydrolase (EC 3.1.4.4, phospholipase D) catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline (1). The enzyme is routinely assayed by single-point determinations using radioactive and nonradioactive substrates (1–3). Phospholipase D can also be assayed by measuring the release of choline through the action of choline oxidase to form hydrogen peroxide. The hydrogen peroxide may be measured amperometrically (4) or by reaction with peroxidase to form a dye complex which absorbs visible light (5,6). These assays do not measure phospholipase D activity continuously and are unsuitable for the detailed kinetic analysis of the enzyme because initial rates of reaction are not necessarily measured. We have developed a recording spectrophotometric assay for the measurement of phospholipase D activity based on the release of choline from phosphatidylcholine which is then coupled via the reactions (below) catalyzed by choline kinase, pyruvate kinase, and lactate dehydrogenase, respectively, to the ultimate formation of NAD^+ which is measured by the decrease in absorbance at 340 nm. This assay allows the continuous monitoring of phospholipase D activity and the determination of initial reaction rates.

[1] To whom correspondence should be addressed.

$$\text{phosphatidylcholine} \xrightarrow[Ca^{2+}]{\text{phospholipase D}} \text{phosphatidic acid} + \text{choline}$$

$$\text{choline} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{choline kinase}} \text{phosphocholine} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[Mg^{2+},\ K^{+}]{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + NAD^{+}$$

MATERIALS AND METHODS

Materials. All chemicals were reagent grade. L-α-Phosphatidylcholine, phosphoenolpyruvate, ATP, NADH, phospholipase D (cabbage), choline kinase, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma Chemical Company. Triton X-100 was a product of Rohm and Haas Company. Phosphatidyl[*N-methyl*-^{14}C]-choline was purchased from Amersham Corporation.

Spectrophotometric assay. Phospholipase D activity was determined by measuring the release of choline from phosphatidylcholine at 30°C by following the decrease in absorbance at 340 nm on a recording Gilford 250 spectrophotometer in the presence of excess NADH, coupling enzymes, and their substrates. The reaction mixture contained 50 mM Tris–maleate buffer (pH 6.5), 0.5 mM phosphatidylcholine, 1 mM Triton X-100, 40 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM KCl, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 10 units of choline kinase, 100 units of pyruvate kinase, 100 units of lactate dehydrogenase, and an appropriate dilution of phospholipase D in a total volume of 0.2 ml. The coupled system was assembled with the exception of phospholipase D. A short incubation was required to eliminate trace amounts of choline or ADP in the reaction mixture. After absorbancy at 340 nm was stabilized, the reaction was initiated by the addition of phospholipase D. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of product/min under the assay conditions described above.

Radioactive assay. Phospholipase D activity was measured by following the release of radioactive choline from phosphatidyl[*N-methyl*-^{14}C]choline (500 cpm/nmol) at 30°C. The reaction mixture was identical to that of the spectrophotometric assay with the exception of omitting the coupling enzymes and their substrates in a total volume of 0.2 ml. The reaction was stopped at 20 min by the addition of 0.5 ml of methanol (0.1 N in HCl). Chloroform (1.5 ml) and 1 M $MgCl_2$ (2 ml) were added, the system was mixed thoroughly, and the phases were separated by a brief centrifugation. An aliquot of the aqueous phase was taken for scintillation counting. A unit of enzyme activity is defined as above.

RESULTS AND DISCUSSION

We have developed a spectrophotometric assay for monitoring the initial rate of reaction of phospholipase D activity utilizing choline kinase, pyruvate kinase, and lactate dehydrogenase as coupling enzymes. The reaction rates of phospholipase D activity using the coupled assay are shown in Fig. 1. An initial lag period (not shown) of about 60 s was followed by a constant rate of NAD^{+} formation which was linear for at least 15 min. The reaction was also linear with enzyme concentration, indicating that initial rates of reaction were being measured (Fig. 2). As little as 0.1 unit of enzyme activity may be determined using the coupled spectrophotometric assay. The sensitivity of the assay is limited by the ability to reliably quantitate NADH oxidation. The radioactive assay is potentially more sensitive since it is limited only by the specific activity of the substrate. The activity of phospholipase D determined

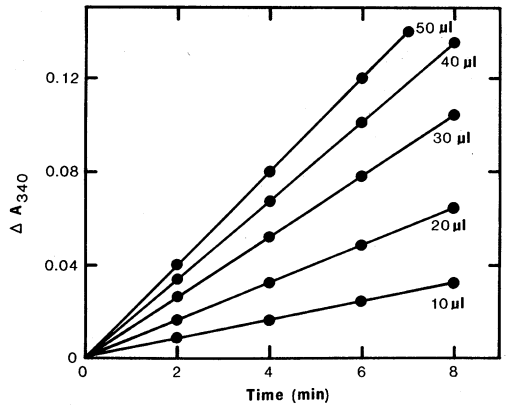

FIG. 1. Time course of the spectrophotometric assay of phospholipase D activity. Phospholipase D contained 11.5 U/ml of activity as determined by the radioactive assay. Each point was taken from a continuous recorder tracing which monitored the disappearance of NADH.

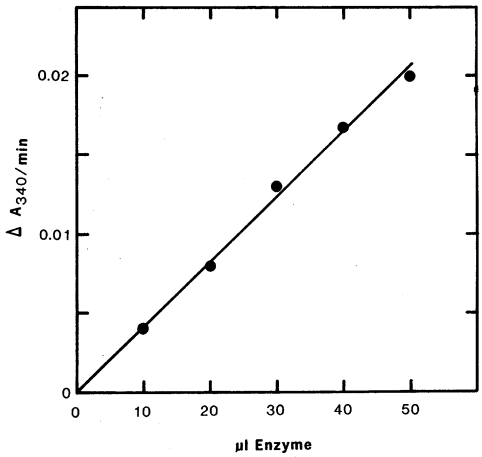

FIG. 2. Enzyme concentration dependence of the spectrophotometric assay of phospholipase D activity. The change in A_{340}/min points was determined from the slopes of each time course plot from Fig. 1.

by the spectrophotometric assay was in good agreement with the activity of the enzyme determined by the radioactive assay (Table 1).

It is important that phospholipase D and the coupling enzymes are free of competing activities to the assay. Such preparations are available commercially at reasonable cost. Crude preparations of phospholipase D containing competing phosphatase and kinase activities are unsuitable for the assay. Therefore such preparations of phospholipase D should be measured for activity by methods reported in Refs. (2–5). However, small background rates of activity due to contaminating activities in a phospholipase D preparation may be corrected for by using a blank with no substrate.

Phospholipase D activity was measured spectrophotometrically with buffers ranging from pH 5.6 to pH 7.0. Optimal activity was found at pH 6.5 with Tris–maleate buffer. The coupled assay for phospholipase D activity was not linear with time when measured at pH 5.6 (the optimum pH for phospholipase D activity (1)). The nonlinearity of the assay may be due to the requirement of higher pH values for activity of the coupling enzymes. However, only about a 5% reduction in activity is observed for the enzyme from rat brain (2) when measured at pH 6.5 rather than at the pH optimum. Therefore, detailed kinetic studies should not be seriously affected by using the coupled spectrophotometric assay.

The coupled spectrophotometric assay is advantageous because it is rapid and measures the initial rate of reaction of phospholipase D activity. The assay is desirable for conducting detailed kinetic studies of the enzyme. In addition, the spectrophotometric assay could potentially be used to

TABLE 1

COMPARISON OF COUPLED SPECTROPHOTOMETRIC ASSAY WITH RADIOACTIVE ASSAY[a]

Assay	Units per milliliter
Spectrophotometric	12.8 ± 1.3
Radioactive	11.5 ± 1.5

[a] Enzyme activity was measured as described under Materials and Methods. Each value is an average of four determinations ± SD.

determine phosphatidylcholine content in biological tissues. Interferences by water-soluble components could be eliminated by a prior phospholipid extraction step.

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